



TITLE:

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A Y-shaped RNA structure in the 3' untranslated region together with the trans-activator and core promoter of *Red clover necrotic mosaic virus* RNA2 is required for its negative-strand RNA synthesis

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Abstract

Red clover necrotic mosaic virus (RCNMV) is a positive-strand RNA virus with a bipartite genome. RNA1 encodes N-terminally overlapping replication proteins, p27 and p88. RNA2 is replicated efficiently by the replication proteins supplied in *trans*, whereas RNA1 needs p88 preferentially in *cis* for its replication. *cis*-Acting elements required for RNA2 replication have been mapped to the 3' terminal stem-loop structure conserved between RNA1 and RNA2, and to the protein-coding region including the trans-activator. Here, we have identified a Y-shaped RNA structure with three-way RNA junctions predicted in the 3' untranslated region of RNA2 as a novel element required for negative-strand synthesis using an *in vitro* translation/replication system. We also show that, in addition to the 3' terminal core promoter, several RNA elements including the *trans*-activator are also required for negative-strand synthesis. Functional roles and structural requirements of these *cis*-acting elements in RCNMV RNA replication are discussed.

Introduction

The genomes of positive-strand RNA viruses are replicated by viral replicase complexes consisting of virus-encoded RNA-dependent RNA polymerase and auxiliary proteins, and possibly host-derived proteins in association with cellular membranes (Ahquist, 2002; Buck, 1996). To accomplish this process, viral replicases must recognize their cognate viral RNAs through direct or indirect interaction with specific sequences or structural elements on the viral RNA template, and recruit them to the replication site to initiate negative-strand RNA synthesis at or near the 3' end of the template genomic RNAs, followed by positive-strand RNA synthesis on the negative-strand RNA (Buck, 1996).

Core promoter elements contain specific sequence and structural features required for recognition by the viral RNA replicase, and have been mapped to the 3' terminal position of viral genomes in many positive-strand RNA viruses including *Red clover necrotic mosaic virus* (RCNMV) (Buck, 1996; Chapman and Kao, 1999;

1 Dreher, 1999; Iwakawa et al., 2007; Panavas et al., 2002; Turner and Buck, 1999;
2 Weng and Xiong, 2009; Zhang et al., 2004). Such RNA elements can be hairpins,
3 pseudoknots, tRNA-like structures (TLSs), or cloverleaf-like structures (Dreher,
4 1999).

5 In addition to the core promoter, positive-strand RNA viruses contain *cis*-acting
6 elements needed for RNA synthesis. These elements are located in the
7 protein-coding regions (Gerber et al., 2001; Goodfellow et al., 2000; Lobert et al.,
8 1999; Mcknight et al., 1998; Panaviene et al., 2005; Park et al., 2002; Tatsuta et al.,
9 2005; Wu et al., 2009; Yang et al., 2008), in the intergenic regions (Chen et al., 2003;
10 Sullivan and Ahlquist, 1999) and in the 5' and 3' untranslated regions (UTRs) of the
11 viral genome (Barton et al., 2001; Dreher, 1999; Nagashima et al., 2005; Sun and
12 Simon, 2006). How the viral replicase recognizes the specific RNA elements
13 including promoters and initiates RNA synthesis is still poorly understood.

14 RCNMV, a member of the genus *Dianthovirus* in the family *Tombusviridae* is a
15 positive-strand RNA virus and its genome is divided into two RNAs, RNA1 and
16 RNA2. RNA1 encodes a 27 kDa protein and its N-terminally overlapping 88 kDa
17 protein that is produced by -1 frameshifting (Kim and Lommel, 1998). Both proteins
18 are required for the replication of RNA1 and RNA2 (Kim and Lommel, 1994; Xiong
19 et al., 1993). RNA1 also encodes a 37 kDa coat protein (CP) that is expressed from a
20 subgenomic RNA (CPsgRNA) (Zavriev et al., 1996). The transcription of CPsgRNA
21 requires an intermolecular interaction between RNA1 and RNA2, in which the
22 *trans*-activator (TA) located in the movement protein (MP)-open-reading frame
23 (ORF) of RNA2 plays a critical role (Sit et al., 1998). The TA also functions as a
24 *cis*-acting element for RNA2 replication (Tatsuta et al., 2005) and as an origin of
25 assembly of RCNMV virions (Basnayake et al., 2009). RNA1 and RNA2 both lack a
26 cap structure at the 5' end and a poly(A) tail at the 3' end (Mizumoto et al., 2002,
27 2003; Xiong and Lommel, 1989). The cap-independent translation enhancer element
28 (3' TE-DR1) of RNA1 resides in its 3' UTR. However, RNA2 has no such element as
29 the 3' TE-DR1. Instead, the cap-independent translational activity of RNA2 is
30 strongly linked to RNA replication (Mizumoto et al., 2006). Therefore, RNA

elements essential for RNA2 replication are also essential to enhance the cap-independent translation of RNA2.

Our previous study has shown that RNA replication mechanisms differ between RNA1 and RNA2. RNA1 replicates preferentially with p88 translated from its own molecule in the presence of p27, whereas RNA2 replicates effectively with p88 supplied in *trans* together with p27 (Okamoto et al., 2008). These results suggest that RNA2 has RNA elements that can interact with replication proteins supplied in *trans*. *cis*-Acting RNA elements required for RNA2 replication have been mapped to the 5' and 3' UTRs (Takeda et al., 2005; Turner and Buck, 1999) and to the MP-ORF (Tatsuta et al., 2005). A terminal stem-loop (SL) structure and another 3' proximal SL are well conserved between RNA1 and RNA2 and among dianthoviruses (Iwakawa et al., 2007). These conserved 3' regions have been shown to be essential for the negative-strand RNA synthesis of RNA1 (Iwakawa et al., 2007) and RNA2 (Takeda et al., 2005; Turner and Buck, 1999; Weng and Xiong, 2009). Functional pseudorecombinants between RNA1 and RNA2 among dianthoviruses (Lommel and Morris, 1982; Okuno et al., 1983) also support the conservation of these RNA elements among dianthoviruses. In contrast to the 3' proximal region, nucleotide sequences in the other region of 3' UTR differ between RNA1 and RNA2. Such regions are also important for negative-strand RNA synthesis of RNA2 (Turner and Buck, 1999) and RNA1 (Iwakawa et al., 2007). Therefore, RNA2-specific RNA elements contained in this region, as well as those in MP-ORF that were previously shown as *cis*-acting elements needed for efficient accumulation of RNA2 in protoplasts, might be candidates that could be involved in different replication mechanisms between RNA1 and RNA2. However, precise functional and structural analyses of RNA elements in the 3' UTR of RNA2 other than the terminal SL (Weng and Xiong, 2009) have not been done.

In this study, we determine RNA elements and their secondary structures required for negative-strand synthesis and replication of RNA2 in detail using an *in vitro* translation/replication system prepared from evacuated-BY2 tobacco protoplast lysate (BYL) and BY-2 protoplasts. We show that novel RNA elements

such as a Y-shaped RNA structure unique to RNA2 are essential for negative-strand RNA synthesis. We also show that SL2 (a main element in TA) and SL6 in the MP-ORF play important roles in negative-strand synthesis, and that the structural requirement of SL2 for RNA synthesis differs from that for the trans-activation of transcription. Functional roles and structural requirements of *cis*-acting RNA elements in RCNMV RNA replication are discussed.

Results

Regions in the 3' UTR required for negative-strand RNA synthesis of RNA2

To delimit the regions in the 3' UTR required for negative-strand RNA synthesis and replication of RNA2, we constructed RNA2 mutants with a series of deletions in the 3' UTR (Fig. 1A), and tested them for the ability to be replicated in the presence of wild type RNA1 that supplies replication proteins in the BYL *in vitro* translation/replication system and BY-2 protoplasts. Total RNAs were extracted from BYL and BY-2 protoplasts after 4 h and 24 h of incubation, respectively. Northern blot analysis showed low accumulation of negative-strand RNAs in several RNA2 mutants (d3, d4, d5, d9, d10, d11, d13, and d14) compared with that of wild-type RNA2 in both BYL and BY-2 protoplasts (Fig. 1B and C). d7 and d8 also showed low accumulations of negative-strand RNA in BYL, whereas much milder or no effects of deletions were observed in BY-2 protoplasts (Fig. 1C). These results suggested that the regions deleted in these RNA2 mutants contained *cis*-acting RNA elements required for negative-strand synthesis.

It should be noted that the accumulation levels of positive-strand RNA in BYL for replication-incompetent RNA2 mutants such as d3, d4, and d5 did not differ from that of wild-type RNA2 (Fig. 1B and C). This result suggests that the accumulation levels of positive-strand RNA in BYL mainly reflect input transcripts. Therefore, the reduced accumulation of positive-strand RNAs observed for d10 and d12 in BYL (Fig. 1B) suggests the importance of the deleted regions for RNA stability.

1

2 *Stem-loop structures other than the terminal SL in the 3' UTR are also important for*
3 *negative-strand RNA synthesis of RNA2*

4

5 To further delimit and characterize RNA elements required for negative-strand
6 synthesis and replication of RNA2, we predicted RNA secondary structures in the 3'
7 UTR of RNA2 (Australian strain) in concert with another RCNMV RNA2 (Canadian
8 strain) using the computer algorithm Dynalign (Mathews and Turner, 2002). In
9 addition to two conserved 3' proximal SL structures (SL11 and the terminal SL)
10 (Iwakawa et al., 2007; this paper), four SLs (SL7, SL8, SL9, and SL10) needed for
11 RNA2 replication are predicted in the regions (Fig. 2A). A structure consisting of
12 SL7 and SL8 with an intervening small SL on a basal SL resembled a Y-shaped
13 structure with three-way junctions that is ubiquitous in RNAs such as riboswitches
14 and ribozymes (De La Pena et al., 2009). This structure was conserved among other
15 dianthoviruses (Fig. 2A). SL10 is also conserved in RNA2 among dianthoviruses,
16 whereas SL9 is not conserved in *Carnation ringspot virus* (CRSV) (data not shown).
17 SL11 is identical to a SL (SLDE) of RNA1 that is essential for negative-strand
18 synthesis of RCNMV RNA1 (Iwakawa et al., 2007).

19 To determine whether these predicted structures are important for RNA
20 replication, first we constructed RNA2 mutants with each of these SLs precisely
21 deleted (Fig. 2B), and tested them in BYL and BY-2 protoplasts as described above.
22 Northern blot results showed that deletion of SL7, SL8, SL10, and SL11, but not
23 SL9, decreased the accumulation of negative-strand RNA2 in both BYL and BY-2
24 protoplasts (Fig. 2C and D). These results suggested the importance of SL7, SL8,
25 SL10, and SL11 in negative-strand RNA synthesis. The reduced accumulation of
26 positive-strand dSL11 compared with other mutants in BYL suggested that SL11 is
27 also important for RNA stability (Fig. 2C).

28

29 *Both SL7 and SL8 and an entire Y-shaped structure are important for*
30 *negative-strand RNA synthesis*

1

2 Next, to determine whether the structures and/or nucleotide sequences of SL7
3 and SL8, and those of the basal stem of the Y-shaped structure are important for
4 negative-strand RNA synthesis, we constructed RNA2 mutants by introducing
5 mutations in the loops and either or both sides of the stems of these predicted
6 structures, which disrupt and restore the stem structures, respectively (Fig. 3A).
7 These mutants were tested in BYL and BY-2 protoplasts as described above.
8 Disruption of the stem structure in either SL7 or SL8 greatly decreased the
9 accumulation of negative-strand RNA2 in the mutants (SL7LM, SL7RM, SL8LM,
10 and SL8RM) compared with that of wild-type RNA2 in both BYL and BY-2
11 protoplasts (Fig. 3B and C), and restoration of the stem structures by compensatory
12 mutagenesis recovered the accumulation of negative-strand RNA2 to wild-type
13 levels in both BYL and BY-2 protoplasts (Fig. 3B and C). Nucleotide substitutions in
14 the loop of SL8 (SL8LoopM), but not SL7, greatly decreased the accumulation of
15 negative-strand RNA2 in BYL and BY-2 protoplasts (Fig. 3B and C). These results
16 indicate that the stem structures of both SL7 and SL8, and the loop sequences of SL8
17 are important for negative-strand accumulation of RNA2.

18 Next, to investigate whether the entire structure of a Y-shaped element is
19 required for the accumulation of negative-strand RNA2, we tested RNA2 mutants
20 with the basal stem of the Y-shaped structure disrupted and restored. Disruption of
21 the basal stem (SL78LM and SL78RM) by nucleotide substitutions upstream of SL7
22 and downstream of SL8 greatly decreased negative-strand RNA accumulations, and
23 restoration of the stem structure by compensatory mutagenesis recovered the
24 accumulation of negative-strand RNA2 to wild-type levels in both BYL and BY-2
25 protoplasts (Fig. 3B and C). These results suggest that the entire Y-shaped structure
26 including SL7 and SL8 is important for negative-strand RNA synthesis of RNA2.

27

28 *The stem structures of SL10 and SL11 and their loop sequences are important for*
29 *negative-strand RNA synthesis*

30

1 To evaluate the structural requirements and the loop sequences of SL10 and
2 SL11 for negative-strand RNA synthesis, we performed mutagenesis analysis of
3 these SLs (see Fig 4A). Disruptions of the stem-structures of SL10 (SL10LM and
4 SL10RM) and SL11 (SL11LM and SL11RM) reduced negative-strand RNA
5 accumulation, and restoration of the structure by compensatory mutations increased
6 RNA accumulation in both BYL and BY-2 protoplasts (Fig. 4B and C). Nucleotide
7 substitutions in the loops of SL10 and SL11 (SL10loopM and SL11loopM) also
8 decreased negative-strand RNA accumulation. Deleterious effects caused by
9 mutations in the loops were much stronger in SL11 than in SL10 (Fig. 4B and C). It
10 should be noted that the accumulation levels of positive-strand RNA of SL11LM,
11 SL11RM, and SL11LoopM in BYL were lower than that of wild-type RNA2,
12 suggesting that the introduced mutations also affected RNA stability. Similar effects
13 on RNA stability were observed for SL10 mutants. These results suggested that
14 SL10 and SL11 are important for negative-strand synthesis, and that these SL
15 structures also are involved in stabilization of RNA2.

16 Similar mutagenesis analyses of the terminal SL13 in BYL and BY-2 protoplasts
17 confirmed the importance of SL13 for negative-strand synthesis (Fig. 4B) (Takeda et
18 al., 2005; Turner and Buck, 1999; Weng and Xiong, 2009).

19

20 *SL2 and SL6 within the coding region are required for efficient negative-strand RNA*
21 *synthesis*

22

23 Our previous study has shown that SL2 and SL6 predicted in the MP-ORF are
24 required for efficient accumulation of RNA2 in cowpea protoplasts (Tatsuta et al.,
25 2005). However, it remained to be addressed whether these elements are required for
26 negative-strand or positive-strand synthesis. To answer this question, we first tested
27 RNA2 mutants that had been used in our previous study including those with SL2 or
28 SL6 deleted, and those with the stem of SL2 disrupted and restored in six
29 nucleotides (Tatsuta et al., 2005) in BYL and BY-2 protoplasts as described above.
30 Northern blot analyses for these mutants showed that the accumulation levels of

1 negative-strand RNA2 and viral RNAs in BYL and in BY2 protoplasts were similar
2 to those observed in cowpea protoplasts (data not shown; Tatsuta et al., 2005),
3 confirming our previous results and suggesting the roles of SL2 and SL6 in
4 negative-strand RNA synthesis.

5 To further investigate the structural features of SL2 required for negative-strand
6 RNA synthesis, we constructed several RNA2 mutants with the stem of SL2
7 disrupted and restored in the upper four, the middle three, or the basal two
8 nucleotides in the stem (Fig. 5A). Effects of the mutations on RNA accumulation
9 were investigated in BYL and BY2 protoplasts as described above. Disruption or
10 deformation of the stem structure by substitution of three and two nucleotides in the
11 middle and basal regions on either side of the stem greatly reduced the accumulation
12 of negative-strand RNA2 in BYL (Fig. 5B). Similar but milder effects of the
13 mutations were observed in BY-2 protoplasts (Fig. 5C). Compensatory mutations to
14 restore the base pairings in these regions partially restored the accumulation of
15 negative-strand RNA2 in both BYL and BY2 protoplasts (Fig. 5B and C).
16 Interestingly, disruption or deformation of the predicted stem structure of SL2 by
17 substitutions in the upper four nucleotides on either side (U4LM and U4RM) had no
18 significant effect on the accumulation of negative-strand RNA2 in both BYL and
19 BY-2 protoplasts (Fig. 5B), unlike other mutations that resulted in deleterious effects
20 on negative-strand RNA accumulation and RNA replication. These results suggested
21 that the two original base pairs at the bottom of the SL2 stem were sufficient to keep
22 a functional conformation of SL2 in negative-strand RNA synthesis and RNA
23 replication. Interestingly, however, restoration of the stem structure by compensatory
24 mutations in the upper four nucleotides in SL2 abolished or greatly reduced the
25 accumulation of negative-strand RNA2 in both BYL and BY-2 protoplasts (Fig. 5B
26 and C). This result was consistent with our previous result that the restoration of the
27 stem structure of SL2 by substitution of six nucleotides only partially recovered its
28 biological activities in cowpea and BY-2 protoplasts (Tatsuta et al., 2005; data not
29 shown). These results suggest that the compensatory mutation to simply restore base
30 pairings in the stem could cause deleterious effects on the configuration of SL2

structure required for RNA replication.

Our previous study has shown that three base substitutions in the loop sequences of SL2 reduced the accumulation of the RNA mutant (TA2) to 50% of that of wild-type RNA2 in cowpea protoplasts, and that this reduction is not caused by the lack of TA-mediated interaction between RNA1 and RNA2 (Tatsuta et al., 2005). To investigate the role of nucleotide sequences in the loop of SL2 in negative-strand RNA synthesis, we tested two RNA2 mutants (TA2 and SL2loopM). SL2loopM has eight base substitutions in the loop sequences of SL2 (Fig. 5A). Eight and three base substitutions in the loop sequences of SL2 abolished or greatly reduced the accumulation of negative-strand RNA2 in both BYL and BY-2 protoplasts (Fig. 5B and C; data not shown). The accumulation levels of negative-strand TA2 was less than 50% of wild-type RNA2 in BYL. These results strongly suggest the involvement of the loop-sequences of SL2 in negative-strand synthesis of RNA2, although eight-nucleotide-long substitution in the loop of SL2 might also affect the configuration of SL2 structure.

Structural requirement of SL2 for RNA synthesis differs from that for trans-activation of transcription

The absence of deleterious effects on RNA replication following disruption of the SL2 stem in the upper four nucleotides prompted us to determine whether U4LM and U4RM have the ability to interact with RNA1 and produce CP through the activation of CPsgRNA transcription from RNA1. To evaluate this ability of U4LM and U4RM, we analyzed CP accumulation using the same BY-2 protoplast samples that were used to evaluate RNA accumulation (Fig. 5). Proteins extracted from the protoplasts were subjected to immunoblot analysis using RCNMV CP antibody. Surprisingly, CP accumulation was below detectable levels in the protoplasts (Fig. 5C) in which U4LM and U4RM accumulated to wild-type levels (Fig. 5B). These results suggested that SL2 with the stem disrupted in the upper four nucleotides did not function as the *trans*-activator of transcription.

Discussion

In this study, we examined RNA elements needed for negative-strand RNA synthesis and replication of RCNMV RNA2 in BYL and BY2 protoplasts, and identified several novel RNA elements such as a Y-shaped RNA structure and SL10 in the 3' UTR of RNA2. We also showed that SL2 and SL6 in the MP-ORF are needed for early RNA replication steps including negative-strand RNA synthesis, and that two original base pairs at the bottom of SL2 stem were sufficient to keep the functional configuration of SL2 required for RNA replication but not for the trans-activation of transcription.

Y-shaped element

A Y-shaped RNA structure is predicted in the 5' half of the RNA2 3' UTR. The Y-shaped structure consists of SL7, SL8 and a short intervening region between them on the basal stem structure. Functional analyses of the RNA elements in the structure by mutagenesis suggest that the entire Y-shaped structure including the structures of SL7 and SL8 and the loop sequences of SL8 are important for negative-strand RNA synthesis. The importance of the Y-shaped structure is supported by the conservation of the structure among dianthoviruses, despite nucleotide sequences within the structures not being conserved so much among the viruses, especially in CRSV (Fig. 2A). The importance of the loop sequence of SL8, but not SL7, in negative-strand RNA synthesis may also be supported by the conservation of the SL8 loop sequence among dianthoviruses. The loop sequences of CRSV SL7 differ from those of RCNMV and SCNMV (Fig. 2A).

We assume that the Y-shaped RNA structure of RCNMV RNA2 functions as a binding site for replication proteins, because our preliminary experiments using strepto-tag affinity binding assay have shown that the 84-nucleotide Y-shaped RNA element is sufficient to interact with p27 in BYL (H. Iwakawa and T. Okuno,

unpublished results). The RCNMV Y-shaped element may resemble a hairpin H4 of TCV, which binds TCV replication protein with greater affinity than the 3' terminal core promoter (Sun and Simon, 2006). The Y-shaped RNA element could be involved in recruiting RCNMV RNAs to replication sites on the membrane, or even in switching between replication and translation in concert with other factors including RNA elements, because cap-independent translational activity of RNA2 is strongly linked to RNA replication (Mizumoto et al., 2006).

RNA elements in the protein coding region

Our results clarified the role of SL2 in negative-strand RNA synthesis, and confirmed the importance of its loop sequences. The formation of base pairings with two original base pairs at the bottom of the SL2 stem was sufficient to maintain a wild-type level function in RNA replication. Compensatory mutagenesis to restore the stem structure through the SL2 stem almost eliminated or greatly reduced its function in negative-strand RNA synthesis (Fig. 5). These results suggest that a precise SL2 configuration including conformational flexibility is important for its function in RNA replication, as discussed below.

SL2 is a multifunctional RNA element: SL2 is a critical factor for the *trans*-activation of transcription through interaction with RNA1 (Sit et al., 1998), and an essential factor required for packaging of genomic RNAs including co-packaging of RCNMV RNA1 and RNA2 (Basnayake et al., 2009). Interestingly, the structural requirement of SL2 for RNA synthesis differs from that for the *trans*-activation of transcription (Fig. 5). Structural changes in SL2 may be important for a proper regulation of one or more steps in the RNA replication process. The U:G base pair at the top of the SL2 stem could be a key element for the potentiality of SL2 structural change. It was reported that the breaking of this U:G base-pair causes the most significant structural change in the 34 nt TA including SL2 in NMR experiments (Guenther et al., 2004). Mutations including a substitution from the U:G base pair to a G:U base pair at the top of the SL2 stem cause

deleterious effects on RNA2 replication (Tatsuta et al., 2005; this report), and on the *trans*-activator function (Guenther et al., 2004).

SL2 locates 600 nucleotides upstream from the 3' end. In terms of its position, SL2 may resemble an internal replication element named RII in the tomato bushy stunt virus genome. RII can specifically bind with a replicase component protein p33, and the interaction of p33 with another replication protein p92 facilitates replicase assembly and recruitment of the genome to the replication site (Pogany et al., 2005). However, it is unlikely that SL2 serves as a binding site for replication proteins because, as mentioned above, our preliminary experiments using strepto-tag affinity binding assays have shown that only RNA fragments containing the Y-shaped structure (84 nt) interact with replicase proteins including p27 and/or p88 (H. Iwakawa and T. Okuno, unpublished results). Instead, it is possible that SL2 interacts with host proteins that are involved in the replication of RNA2.

RNA2-specific RNA elements identified here are possible candidates that could elucidate different replication and translation mechanisms between RNA1 and RNA2. These elements required for RNA replication might be involved even in switching between replication and translation, because cap-independent translational activity of RNA2 is strongly linked with RNA replication (Mizumoto et al., 2006). Further studies incorporating viral and host proteins are under way to elucidate RCNMV replication processes, in which the RNA elements identified here perform their biological activities.

MATERIALS AND METHODS

Plasmid clones and their construction

pUCR1 and pRC2|G are full-length cDNA clones of RNA1 and RNA2 of RCNMV Australian strain, respectively (Takeda et al., 2005; Xiong and Lommel, 1991). pRNA2fsMP is a cDNA clone of an RNA2 mutant whose transcripts replicate as efficiently as wtRNA2, but do not express intact MP (Tatsuta et al., 2005). All

1 mutations in pRC2|G and pRNA2fsMP, were introduced by using polymerase chain
2 reaction (PCR)-based mutagenesis with primers listed in Table 1. Each construct was
3 sequenced across its entire PCR-derived region to ensure that only the desired
4 mutation was present. Details of the modified RNA sequence and/or structure are
5 presented in the figures.

6 Plasmid clones to transcribe RNA2 mutants with mutations in the 3' UTR were
7 constructed using pRC2|G. To construct these clones except for those to transcribe
8 d14 and SL78LRM, the primer pairs used were R2-934+ plus one each of the
9 following: d1-, d2-, d3-, d4-, d5-, d6-, d7-, d8-, d9-, d10-, d11-, d12-, d13-, dSL7-,
10 dSL8-, dSL9-, dSL10-, dSL11-, SL7LM-, SL7RM-, SL7LRM-, SL7loopM-,
11 SL8LM-, SL8RM-, SL8LRM-, SL8loopM-, SL78LM-, SL78RM-, SL10LM-,
12 SL10RM-, SL10LRM-, SL10loopM-, SL11LM-, SL11RM-, SL11LRM-,
13 SL11loopM-, SL13LM-, SL13RM-, SL13LRM-, and SL13loopM-, respectively.
14 Another primer, R2-1540-, was used together with one each of the following: d1+,
15 d2+, d3+, d4+, d5+, d6+, d7+, d8+, d9+, d10+, d11+, d12+, d13+, dSL7+, dSL8+,
16 dSL9+, dSL10+, dSL11+, SL7LM+, SL7RM+, SL7LRM+, SL7loopM+, SL8LM+,
17 SL8RM+, SL8LRM+, SL8loopM+, SL78LM+, SL78RM+, SL10LM+, SL10RM+,
18 SL10LRM+, SL10loopM+, SL11LM+, SL11RM+, SL11LRM+, SL11loopM+,
19 SL13LM+, SL13RM+, SL13LRM+, and SL13loopM+, respectively. The amplified
20 DNA fragments were mixed together and further amplified by PCR using the primer
21 pair R2-934+ and R2-1540-, digested with *Xba*I and *Sma*I, and inserted into the
22 corresponding region of pRC2|G.

23 To construct a plasmid clone for transcribing d14, cDNA fragments were
24 amplified by PCR from pRC2|G using a primer pair R2-934+ plus d14-, and the
25 amplified DNA fragments were digested with *Xba*I and *Sma*I, and inserted into the
26 corresponding region of pRC2|G.

27 To construct a plasmid clone for transcribing SL78LRM, three cDNA fragments
28 were amplified by PCR from pRC2|G using three sets of primer pairs, R2-934+ plus
29 SL78LM-, SL78LM+ plus SL78RM-, and SL78RM+ plus R2-1540-. The amplified
30 DNA fragments were mixed, and further amplified by PCR using the primer pair

R2-934+ and R2-1540-. The amplified DNA fragments were digested with *Xba*I and *Sma*I, and inserted into the corresponding region of pRC2|G.

Plasmid clones to transcribe RNA2 mutants with mutations in the MP ORF were constructed using pRNA2fsMP. The primer pairs used were R2-260+ plus one each of the following: U4LM-, U4RM-, U4LRM-, SL2loopM-, M3LM-, M3RM-, M3LRM-, B2LM-, B2RM-, B2LRM-, respectively. Another primer, R2-1360-, was used together with one each of the following: U4LM+, U4RM+, U4LRM+, SL2loopM+, M3LM+, M3RM+, M3LRM+, B2LM+, B2RM+, B2LRM+, respectively. Recombinant PCR products were amplified with the primer pair R2-260+ plus R2-1360-, digested with *Nhe*I and *Nco*I, and inserted into the corresponding region of pRNA2fsMP.

RNA preparation

All RNA transcripts were synthesized *in vitro* from *Xma*I-linearized plasmids with T7 RNA polymerase and purified with a Sephadex G-50 fine column (GE Healthcare Bio-Sciences Corp., Piscataway NJ) (Mizumoto et al. 2006). Transcript concentrations were measured by spectrophotometer, and its integrity was verified by 1% agarose gel electrophoresis.

Protoplast experiments

Protoplast experiments using tobacco BY-2 suspension-cultured cells were performed as described previously (Takeda et al., 2005; Iwakawa et al., 2007). Washed protoplasts were resuspended in cold MES buffer (0.6 M Mannitol, 5 mM MES, 5 mM CaCl₂, pH 5.7) at 1.67×10^6 cells/ml. Wild-type RNA1 (1.1 pmol) and wild-type RNA2 or RNA2 mutants (2.9 pmol) were suspended in 0.2 ml of cold MES buffer and mixed with 0.6 ml of protoplast solution before electroporation (125 μ F capacitance, 300 V, 100 Ω) using a Pulse Controller Plus (Bio-Rad). Protoplasts were incubated at 17 °C for 24 h in the dark.

Evacuolated BY-2 protoplast lysate (BYL) experiments

Preparation of BYL and *in vitro* translation/replication reaction were described previously (Iwakawa et al., 2007; Komoda et al., 2004).

Northern blot analysis

Total RNAs extracted from BY-2 protoplasts and BYL were subjected to Northern blot analysis as described previously (Iwakawa et al., 2007). The digoxigenin (DIG)-labeled RNA probes specific for RCNMV RNA2 3' UTR and negative-strand RNA2 were prepared as described previously (Iwakawa et al., 2007; Mizumoto et al., 2002). The RNA signals were detected with a luminescent-image analyzer (LAS 1000 plus; Fuji Photo Film), and the signal intensities were quantified with the Image Gauge program (Fuji Photo Film).

Western blot analysis

Western blot analysis was performed as described previously (Tatsuta et al., 2005). The signals were detected with a luminescent-image analyzer (LAS 1000 plus; Fuji Photo Film) and the signal intensities were quantified with the Image Gauge program (Fuji Photo Film).

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- 19

FIGURE LEGENDS

Fig. 1. Effects of deletions in the 3' UTR on the accumulations of positive- and negative-strand RCNMV RNA2 in BYL and BY-2 protoplasts. (A) Schematic representation of deleted regions in the 3' UTR of RCNMV RNA2. Thick horizontal lines indicate virus-derived sequence with the nucleotide numbers at the 5' and the 3' ends. Thin bent lines indicate deleted regions. (B) Accumulations of positive- and negative-strand RNA2 in BYL incubated with wtRNA2 or RNA2 mutants with a series of deletions in the 3' UTR together with wtRNA1. Total RNAs were extracted after 4 h of incubation, and analyzed by Northern blotting. The ribosomal RNA (rRNA) is shown below the Northern blots as a loading control. (C) Accumulations of positive- and negative-strand RNA2 in BY-2 protoplasts inoculated with wtRNA2 or RNA2 mutants together with wtRNA1. Total RNA was extracted after 24 h of incubation. rRNA is shown below the Northern blots.

Fig. 2. (A) Secondary RNA structures predicted in the regions, in which deletions caused deleterious effects on the accumulation of RCNMV RNA2 (see Fig. 1). Secondary RNA structures were predicted using the computer algorithm Dynalign (Mathews and Turner, 2002). Y-shaped RNA structures, SL10s and SL11s predicted in *Sweet clover necrotic mosaic virus* (SCNMV) and CRSV are presented above those of RCNMV. (B) Schematic representation of deleted regions in the 3' UTR of RCNMV RNA2. Thick horizontal lines indicate virus-derived sequences with the nucleotide numbers at the 5' and 3' ends. Thin bent lines indicate deleted regions corresponding to each stem-loop structure. (C and D) Accumulations of positive- and negative-strand RNA of wtRNA2 or RNA2 mutants with each of the predicted SL structures deleted in BYL (C) and BY-2 protoplasts (D) after incubation or inoculation together with wtRNA1. For others, refer to the legend of Fig. 1.

Fig. 3. Effects of mutations in SL7, SL8, and the basal stem of the Y-shaped structure on accumulations of positive- and negative-strand RNA2 in BYL and BY-2

protoplasts. (A) Schematic representations of the Y-shaped structure with mutations. Boldface italics in boxes show disrupted and restored stem structures of SL7 and SL8 together with the basal stem and altered loop sequences in SL7 and SL8. (B and C) Accumulations of positive- and negative-strand RNA of wtRNA2 or RNA2 mutants in BYL (B) and BY-2 protoplasts (C) after incubation or inoculation together with wtRNA1. For others, refer to the legend of Fig. 1.

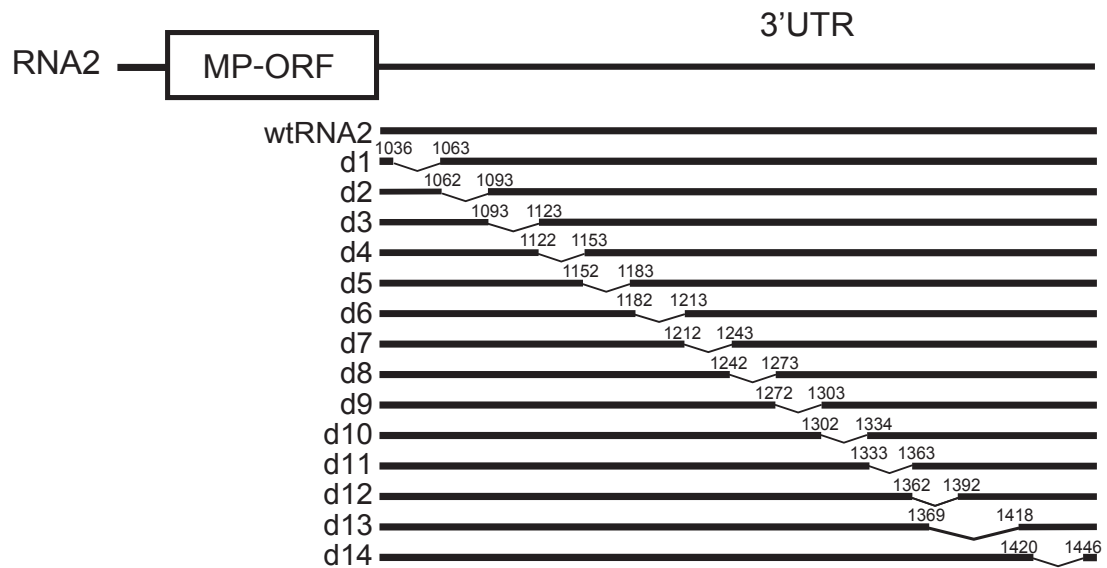
Fig. 4. Effects of mutations in SL10, SL11 and SL13 on accumulations of positive- and negative-strand RNA2 in BYL and BY-2 protoplasts. (A) Schematic representations of SL10, SL11 and the terminal SL13 with mutations. Boldface italics in boxes show disrupted and restored stem structures and altered loop sequences. (B and C) Accumulations of positive- and negative-strand RNA of wtRNA2 or RNA2 mutants in BYL (B) and BY-2 protoplasts (C) after incubation or inoculation together with wtRNA1. For others, refer to the legend of Fig. 1.

Fig. 5. Effects of mutations in SL2 on accumulations of positive- and negative-strand RNA2 in BYL and BY-2 protoplasts. (A) Schematic representation of RNA2fsMP (Tatsuta et al., 2005) with introduced mutations. Boldface italics in boxes show disrupted and restored stem structures of SL2 in the upper four, the middle three, or the basal two nucleotides in the six base pairs of the stem, and altered loop sequences. (B and C) Accumulations of positive- and negative-strand RNA of wtRNA2 or RNA2 mutants in BYL (B) and BY-2 protoplasts (C) after incubation or inoculation together with wtRNA1. For U4 mutants and SL2loop mutant, we performed Western blot analysis of CP accumulation in BY-2 protoplasts inoculated with wtRNA1 and RNA2 mutants. CP was detected using an anti-RCNMV-CP antibody. Coomassie brilliant blue-stained cellular protein is shown below the western blots as a loading control. For others, refer to the legend of Fig. 1.

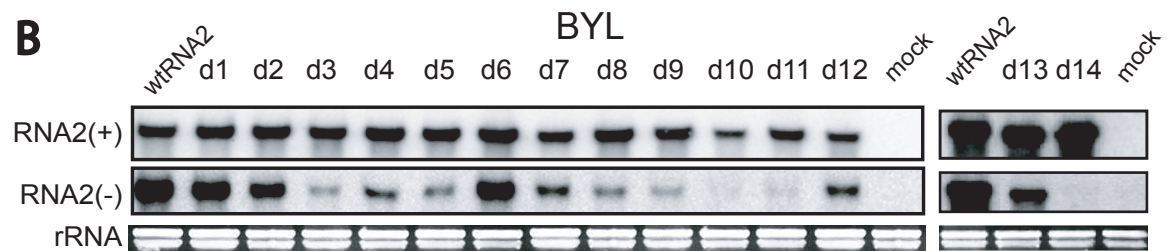
Table 1
Primers used for PCR to generate constructs

Primers	Sequence	Primers	Sequence
d1+	GGAAAGACTCCACTGAAAAAGTGGAATCTC	78L+	AAGAGATTCTGAATTGCTTTGGCTTCGGC
d1-	TTTTTCAGTGGAGTCTTTCGGATTGGGTC	78L-	GCAATTCAGAATCTCTTTGTACTCTGGAT
d2+	GATGCGTGGCCAAAGAGAAAGAGAATTGC	78R+	AGGGTCTCAGAATCTTGGCCTTTAGTGGGC
d2-	TTCTCTTTTGGCCACGCATCTGACTTCCCC	78R-	AAAGGCCAAGATTCTGAGACCCTACGAGGA
d3+	ATCCAGAGTAGCGGAAGACGAAAAGGTTAAATAC	SL10LM+	GATTTGGACCCGTTGGTCCCTCCCGGTGAG
d3-	CGTCTTCCGCTACTCTGGATGAGATTCC	SL10LM-	GACCAACGGGTCCAAATCTCTCGAAAGTAAC
d4+	TTTGGCTTCGACGAGTTCTCCTCGTAGGGTC	SL10RM+	GGTTGGTGGGTCCCGGTGAGAGAAGAAAAC
d4-	GAGAACTCGTCGAAGCCAAAGCAATTCTC	SL10RM-	ACCGGGACCCACCAACCCCTCCAAATC
d5+	AATACAGGCTTGGCCTTTAGTGGGCTTTGG	SL10LRM+	GGACCCGTTGGTGGGTCCCGGTGAGAGAAGAAAAC
d5-	CTAAAGGCCAAGCCTGTATTTAAACCTTTTC	SL10LRM-	GACCCACCAACGGGTCCAAATCTCTCGAAAGTAAC
d6+	CTCTCTTTCTATTTCTACAGCGTATGGACC	SL10loopM+	GGGGAACCTCCCTCCCGGTGAGAGAAGAAAAC
d6-	CTGTAGAAATAGAAAGAGAGACCCTAC	SL10loopM-	GGGAGGGAGGTTCCCTCCAAATCTCTC
d7+	TTTAGGTTCTTCAGCCTATCGTAGTGTGTGC	SL11LM+	CCCGGACTGAGAAGAAAACCTATCC
d7-	ATAGGCTGAAGGAACCTAAACCAAAGCCC	SL11LM-	TTTTCTTCTCAGTCCGGGAGGGACCAAC
d8+	ATGGATGTTCTCGGTTAGGGGTTACTTTC	SL11RM+	AAAACAGTTCCAAACAAAAACAGGGTTTC
d8-	CCCTAACGCAGAACATCCATGGTCCATAC	SL11RM-	TTTGTTTGGAAGTCTTTCTCTCTCACC
d9+	GCGATAGCATAGGGGTTGGTCCCTCCC	SL11LRM+	ACTGAGAAGAAAACAGTTCCAAACAAAAACAGGGTTTC
d9-	ACCAACCCCTATGCTATCGCACACACTAC	SL11LRM-	GGAACTGTTTTCTTCTCAGTCCGGGAGGGACCAACCCCTC
d10+	AGAGATTGGACTCATCCAAACAAAAAAC	SL11loopM+	TGAGTCTTCTTTTCTCATCCAAACAAAAAAC
d10-	TTGGATGAGTCCAAATCTCTCGAAAGTAAC	SL11loopM-	TGGATGAGAAAAGAAGACTACCCGGGAGGGACCAAC
d11+	AGAGAAGAAAGTAACCGGTGCGACAATATGC	SL13LM+	TAATGCCATCTCGTATAACGGCTAGGCAC
d11-	CACCGGTTACTTTCTTCTCTCACCGGGAGGGAC	SL13LM-	TACGAGATGGCATTATAGTTTTTCTCC
d12+	AGGGTTTCTGGCACTGACCGGCACGGAGAAAAAC	SL13RM+	AACGGGATGGCACCCCGGGATGCAAGC
d12-	CGGTCACTGTCAGAAACCCCTGTTTTTGGT	SL13RM-	TCCCGGGGTGCCATCCCGTTATACGACTAGGC
d13+	TCTGTAACCGTAATGCCTAGTCGTATAAC	SL13LRM+	CCATCTCGTATAACGGGATGGCACCCCGGGATGCAAGC
d13-	ACTAGGCATTTCGTTACAGAAACCCCTGTTT	SL13LRM-	CCATCCCGTTATACGAGATGGCATTATAGTTTTCTCC
d14-	GCATCCCGGGGTTATAGTTTTTCTCCGTGCCGG	SL13loopM+	AGTCGTTATACGGCTAGGCACCCCGGGATGC
dSL7+	GAAAGAGAAAAAGGTTTAAATACACGCTAC	SL13loopM-	AGCCGTATATCGACTAGGCATTATAGTTTTTC
dSL7 -	TTTAAACCTTTTTCTCTTCTCTTTGTAC	U4LM+	TCAATCAGTCCGATCGCCCCGCCTCTC
dSL8 +	TAAATACAGGGGTCTCTTCTCTTGGCC	U4LM-	GGCGATCGGACTGATTGAACCTGTCTC
dSL8 -	AAGAGAGACCCCTGAATTAAACCT TTTC	U4RM+	TATCGCCCTGGACTCAGTGTGTACCAAAC
dSL9 +	GCGATAGCATAGGGGTTGGTCCCTCCC	U4RM-	CTGAGTCCAGGGGCGATACCTCTGATTGAAC
dSL9 -	ACAACCCCTATGCTATCGCACACACTAC	U4LRM+	CAGTCCGATCGCCCCCTGGACTCAGTGTGTACCAAAC
dSL10 +	CGAGAGATTTCGGTGAGAGAAGAAAACTC	U4LRM-	GAGTCCAGGGGCGATCGGACTGATTGAACCTGTCTCTGC
dSL10 -	TCTCTACCGAAATCTCTCGAAAGTAACCC	SL2loopM+	AGAGGTTAGCGGGGGCCTCTCAGTGTGTGACC
dSL11 +	GGTCCCTCCCCAAACAAAAACAGGGTTTC	SL2loopM-	GAGGCCCCCGCTAACCTCTGATTGAACCTGTC
dSL11 -	TTTTTGTGGGGAGGGACCAACCCCTCC	M3LM+	AGGTTCAATCACTCGTATCGCCCCGCCTC
SL7LM+	AATTGGAATGGCTTCGGCGGAAGAC	M3LM-	GGGCGATACGAGTGATTGAACCTGTCTCTGC
SL7LM-	CGAAGCCATTCCAATTCTCTTTCTC	M3RM+	GCCCCGCGAGTCAGTGTGTACCAAAC
SL7RM+	GGCGGTTACGAAAAGGTTTAAATAC	M3RM-	TACAACACTGACTCGCGGGGCGATACCTC
SL7RM-	CITTTCTGTGAAGCCGCCGAAGCCAAAGC	M3LRM+	CTCGTATCGCCCCGCGAGTCAGTGTGTACCAAACCAAGGAC
SL7LRM+	TGGAATGGCTTCGGCGGTTACGAAAAGGTTTAAATAC	M3LRM-	CTCGCGGGGCGATACGAGTGATTGAACCTGTCTCTGCTT
SL7LRM-	GAACCGCCGAAGCCATTCCAATTCTCTTTCTC	B2LM+	AGGTTCAATCTCAGGTATCGCCCCGCCTC
SL7loopM+	TTGGCAAGCGCGGAAGACGAAAAGGTTTAAATAC	B2LM-	GGCGATACCTGAGATTGAACCTGTCTCTGC
SL7loopM-	CGTCTTCCGCGCTTGCCAAAGCAATTCTC	B2RM+	GCCCCGCCTGACAGTGTGTACCAAAC
SL8LM+	GGCTAGCTGTTCTCCTCGTAGGGTC	B2RM-	TACAACACTGTACGGCGGGGCGATACCTC
SL8LM-	AGGAGAACAGCTAGCCTGTATTTAAAC	B2LRM+	TCAGGTATCGCCCCGCCTGACAGTGTGTACCAAACCAAGGAC
SL8RM+	GTTCTCCAGCTAGGGTCTCTCTTTTC	B2LRM-	TCAGGCGGGGCGATACCTGAGATTGAACCTGTCTCTGCTTT
SL8RM-	AGACCTAGCTGGAGAACTCGTAGCC	R2-934+	CTCACAGAAATGTTCTGAAC
SL8LRM+	GGCTAGCTGTTCTCCAGCTAGGGTCTCTCTTTTC	R2-1540-	GTTGTGTGGAATTGTGAGCG
SL8LRM-	CCCTAGCTGGAGAACAGCTAGCCTGTATTTAAAC	R2-260+	TGTTGCGCTTAATTACACCCAC
SL8loopM+	ACGAGAAGAGCTCGTAGGGTCTCTC	R2-1360-	TTTCTTCTCTACCCGGGAGGGAC
SL8loopM-	ACGAGCTCTTCTCGTAGCCTGTATTTAAAC		

A



B



C

